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Patterned Hydrophobic Domains in the Exopolymer Matrix of *Shewanella oneidensis* MR-1 Biofilms

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Water-dispersible amphiphilic surface-engineered quantum dots (QDs) were found to be strongly accumulated within discrete zones of the exopolymer network of *Shewanella oneidensis* MR-1 biofilms, but not on the cell surfaces. These microdomains showed a patterned distribution in the exopolymer matrix, which led to a restricted diffusion of the amphiphilic nanoparticles.

The self-produced matrix of organic exopolymeric substances (EPS) contributes to biofilm three-dimensional (3D) architecture and stability. It is composed of proteins capable of carrying out enzymatic activities, polysaccharides, lipids, nucleic acids, and heteropolymers (1–4). The EPS entanglement is mostly hydrated (5), and its cohesiveness is governed by many interactions, including hydrogen bonding, cross-linking by multivalent cations, and hydrophobic associations (6). These interactions dramatically influence the process of diffusion of ions, molecules, and particles through the three-dimensional organization of the biofilm framework (7–11). The EPS matrix behaves as hydrophobically modified hydrogels (12), and the distribution of the hydrophobic regions of the matrix should be quite heterogeneous, as pointed out by previous reports (3, 11). It is suspected that these hydrophobic domains contribute to the accumulation of poorly soluble compounds (13–15) and to the adhesion of hydrophobic cells (16). Their distribution in the EPS matrix and/or on the cell surfaces is, however, still an open question.

Recently we have reported the use of amphiphilic CdSe/ZnS (core/shell) quantum dots (QDs) capped with dihydrolipoic acid (DHLA) linked to phenylalanine amino acid (CdSe/ZnS–DHLA-Phe) for the exploration of the hydrophilic/hydrophobic balance in bacterial biofilms (17). We found that the heterogeneous distribution of these QDs within the biofilm depended on their surface amphiphilicity without demonstrating their exact localization. Here, we built on those findings and report the use of these red-emitting amphiphilic QDs to explore their interaction with planktonic cells and their localization within *Shewanella oneidensis* biofilms. These amphiphilic QDs were used to target unambiguously the presence of hydrophobic domains in the EPS of biofilms and show the heterogeneity of these compartments organized under the control of living bacterial cells. For this purpose, the amphiphilic QD diffusion was analyzed noninvasively by fluorescence correlation spectroscopy (FCS) and QD accumulation was imaged by laser scanning confocal microscopy (LSCM).

Amphiphilic QDs did not interact with planktonically grown *Shewanella oneidensis* cell envelopes. *Shewanella oneidensis* MR-1, used in this study, belongs to a genus represented by many members, whose surface properties (charge, hydrophobicity) may differ significantly according to the species or the environment (18). The *Shewanella* genus is ubiquitous; it has been found in marine water and freshwater (Lake Oneida, NY) and associated with sediments and is known to form biofilms (19). Moreover, it represents an ideal candidate for our study because of its aerobic and anaerobic physiology, which may reinforce its adaptation to biofilm growth and activity stratification.

S. oneidensis MR-1 cells were grown at 30°C for 18 h in nutritive medium supplemented with lactate and fumarate (LML+F) and washed twice by centrifugation/suspension in $MgSO_4$ solution (10 mM). We ascertained by FCS measurements that the red-emitting CdSe/ZnS–DHLA-Phe QDs (hydrodynamic diameter, ~20 nm) did not interact with the cell surface of these laboratory-grown bacteria (Fig. 1). FCS experiments were performed on an SP5 (Leica Microsystems, France) confocal microscope using an excitation wavelength of 488 nm, leading to a photoluminescence centered at 600 nm as detailed in reference 17. The fluorescence autocorrelation curves [$g(\tau)$] were fitted by a two-component diffusion model (7). The QD diffusion times (τ_{1D}) and coefficients (D) in water dispersion and in *S. oneidensis* suspension were quite similar ($\tau_{1D} = 0.55 \pm 0.05$ ms, $D = 2.4 \pm 0.3 \times 10^{-11}$ m^2/s and $\tau_{1D} = 0.64 \pm 0.05$ ms, $D = 2.1 \pm 0.3 \times 10^{-11}$ m^2/s, respectively). The slightly distorted correlation signal observed corresponds to QD aggregates both in water and in bacterial suspensions with respective amounts of 16% and 28% and a mean diffusion time (τ_{2D}) of ~8.0 ± 3 ms. Moreover, neither surface degradation nor loss of fluorescence due to changes in the electronic environment was detected for the amphiphilic QDs used. Thus, the noninteraction of our negatively charged QDs with the planktonic cells could be explained by the presence of hydrophilic capsular polysaccharides (18).

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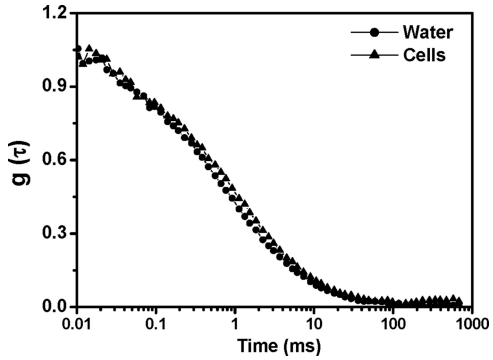

FIG 1 Fluorescence autocorrelation curves [$g(\tau)$] of 250 nM amphiphilic QDs in water and in the presence of planktonic cells.

CdSe/ZnS–DHLA-Phe QDs accumulated in microdomains in the EPS matrix of *Shewanella oneidensis* MR-1 biofilms. The mobility of the amphiphilic QDs in the biofilm was found to be drastically reduced, as revealed by the distortion of the FCS curves measured at different points through the biofilm in comparison with that obtained for QDs dispersed in water (Fig. 2). In addition, the diffusion kinetics of QDs inside the biofilm was monitored by time course fluorescence imaging for 75 min. By assessing the diffusion kinetics of QDs inside the biofilm using time course fluorescence imaging for 75 min, we found that the QDs accumulate in every part of the biofilm, with a more pronounced concentration at the periphery than at the center of bacterial aggregates (see Fig. S1 in the supplemental material), contrary to what has been previously shown with hydrophilic QDs (17).

In order to specify the biofilm parts labeled by the amphiphilic QDs, i.e., extracellular microdomains of *S. oneidensis* MR-1 biofilms and/or cell surface of the bacteria, a dual staining was performed with both 2.5 μM Syto 9, a cell-permeant nucleic acid stain which allows easy observation of the cells, and the QDs. Confocal microscopy imaging was performed using a beam line from a continuous argon ion laser as the sole source of excitation at 488 nm (17). The Syto 9 and CdSe/ZnS–DHLA-Phe QD fluorescence signals were collected at 495 to 530 nm and 580 to 610 nm, respectively. Furthermore, we applied a spectral reassignment procedure based on a Bio-Rad algorithm to reduce the inherent fluorescence overlap of each chromophore. Figure 3, as well as Fig. S2 in the supplemental material, shows clearly that the cells are easy to recognize due to the green fluorescence of Syto 9 and uniformly distributed in the biofilm, while the amphiphilic QDs are unevenly accumulated and form clusters in the extracellular space between the bacterial cells. Some of the tagged areas (see Fig. S2B in the supplemental material) are large (5 to 15 μm). This QD overaccumulation could be partly attributed to probe-probe interactions. According to previous semivariogram calculations carried out on the whole biofilm (17), the pseudoperiodic distribution of the amphiphilic QDs should be attributed to the EPS matrix only. The QD accumulation in the exopolymeric matrix of the biofilm reveals irregularly shaped, micrometric (average extent, ca. 4 ± 2.2 μm), closely spaced (2 to 5 μm side to side), and patterned hydrophobic microdomains (Fig. 4). The frontier of the exopolymeric matrix in Fig. 4 is based on the outline estimated on the basis of the threshold of gray levels of wide-field images. This consistent information suggests a high density of hydrophobic microdomains per unit volume (up to 10^6 per mm^3) of the *S. oneidensis* biofilm matrix. We have also checked the distribution of the hydrophobic domains in biofilms grown under higher (ca. 6 mg/liter) versus lower (ca. 1 mg/liter) dioxygen concentrations, as the concentration of dioxygen has been reported to be one determinant parameter of biofilm cohesiveness (20). However, similar patterns of QD cluster distribution were observed (data not shown), suggesting the same matrix architecture with respect to hydrophobic microdomains even with the lowest dioxygen concentration tested here.

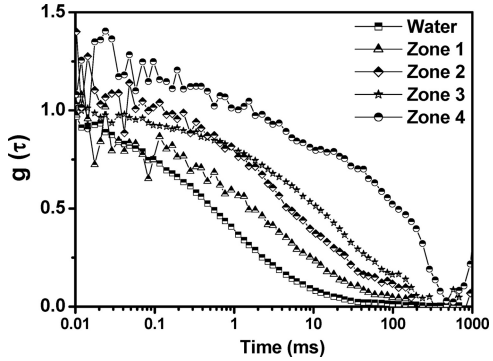

FIG 2 Fluorescence autocorrelation curves [$g(\tau)$] of QDs in four points in the biofilm, measured with respect to a separation distance of 10 μm, and in pure water (the lowest curve).

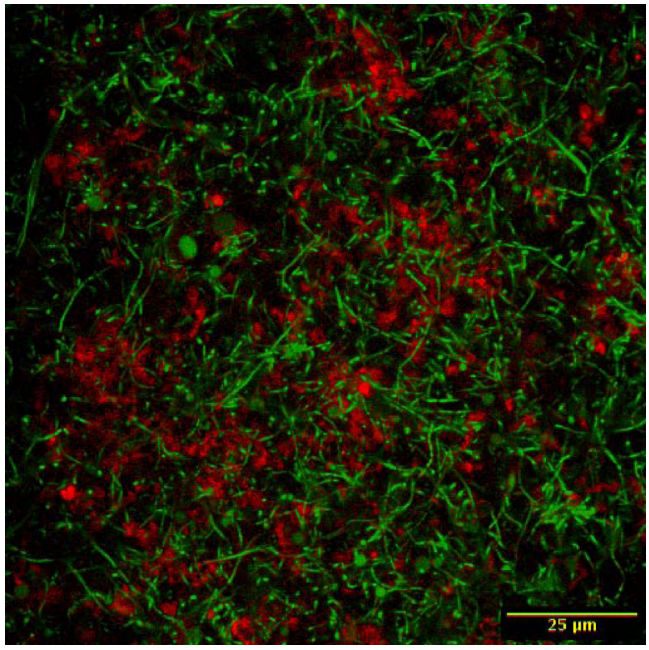

FIG 3 Confocal microscopy image taken at 30 μm from the bottom glass slide after dual staining of a *Shewanella* biofilm with 2.5 μM Syto 9 (green) and 250 nM amphiphilic CdSe/ZnS–DHLA-Phe QDs (red).

To summarize, using a surface-functionalized amphiphilic quantum dot we demonstrated the presence of a high density of hydrophobic microdomains with a patterned distribution throughout the exopolymer matrix of *S. oneidensis* MR-1 biofilms. This hydrophobic texture (i.e., arrangement and size of microdomains) in such a highly hydrated network should allow a protec-

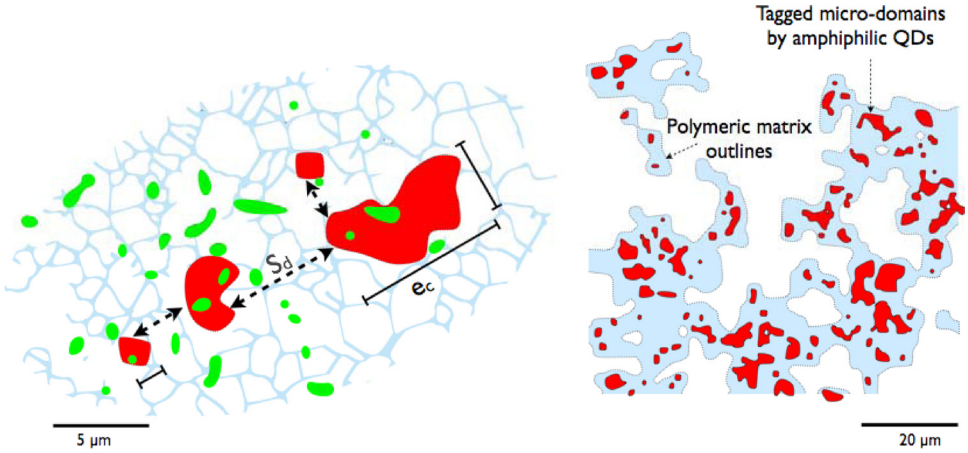

FIG 4 Schematic representation of *Shewanella* biofilm texture showing structure pattern of hydrophobic domains within EPS matrix (blue regions). Clustered hydrophobic domains (red) generate pseudoperiodic structures in the whole polymeric architecture (blue). S_d, average microdomain spacing; e_c, cluster extent. The green objects represent bacterial cells and their distribution.

tive accumulation of poorly soluble xenobiotics (e.g., steroids, hydrocarbons, etc.) outside the cells.

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